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(54) Title: A REAGENT STABILIZED BY BIVALENT METAL SALTS

(57) Abstract

A stable reagent for the analysis of an analyte. The reagent includes an enzyme, a phenazine derivative, a tetrazolium salt, and a divalent metal salt to stabilize the reagent. Importantly, the inclusion of the divalent metal salt in the reagent stabilizes the phenazine derivative/tetrazolium salt system at pHs from about 5.5 to about 7.8, thereby permitting the optimal functioning of certain enzymes in the assay of an analyte. The reagent may be provided in solution form or may be incorporated into a film, a fabric mesh, a glass fiber matrix, or a paper matrix.

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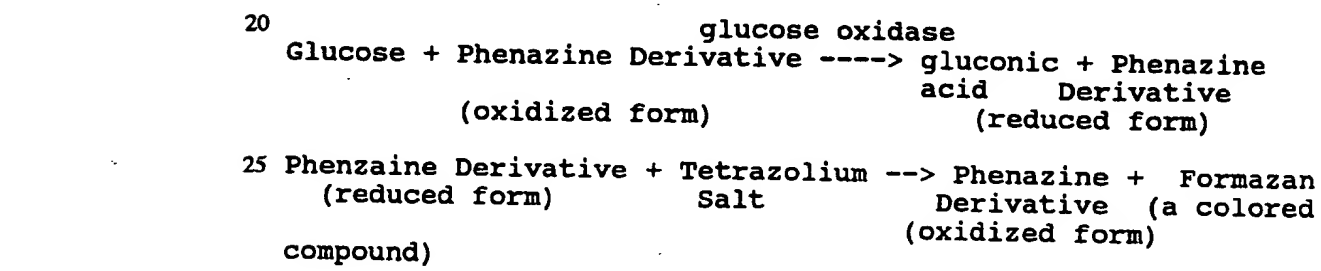
A REAGENT STABILIZED BY DIVALENT METAL SALTS

FIELD OF THE INVENTION

The invention relates to the chemical analysis of an analyte by the formation of a colored indicator.

BACKGROUND OF THE INVENTION

A common way of indirectly measuring the amount of analyte in a sample is to involve the analyte in a sequence of reactions that lead to the generation of color, wherein the intensity of the color generated is proportional to the amount of analyte in the sample. A known method of performing this type of chemical assay utilizes reagents that include a phenazine derivative and a tetrazolium salt (the phenazine derivative/tetrazolium salt system). The phenazine derivative/tetrazolium salt system may be schematically depicted by the following scheme for glucose analysis:



Further illustrations of the phenazine derivative/ tetrazolium salt system in chemical analysis may be found in the following references: Findlay et al., U.S. Patent 4,713,327, issued December 15, 1987; Josef et al., U.S. Patent 3,791,988, issued February 12, 1974; Forgione et al., U.S. Patent 3,929,580, issued December 30, 1975; Shigeta et al., U.S. Patent 4,803,158, issued February 7, 1989; Self, U.S. Patent 4,446,231, issued May 1, 1984; and Self, U.S. Patent 4,598,042, issued July 1, 1986.

A problem with the phenazine derivative/tetrazolium salt system is the stability of the phenazine derivative

and the tetrazolium salt, particularly when the phenazine derivative and tetrazolium salt are included in a single reag nt or a film at pHs above about 5.5. At pHs above about 5.5, photochemical degradation of the phenazine derivative and autoreduction of the tetrazolium salt become significant and interfere with accurate and precise correlation of formazan to the analyte being measured. Because many assays involve the use of enzymes that function best at pHs from about 5.5 to about 7.8, it is desirable to provide means for stabilizing the phenazine derivative/tetrazolium salt system in this pH range.

SUMMARY OF THE INVENTION

The invention is a stable reagent for the analysis of an analyte. The reagent includes an enzyme, a phenazine derivative, a tetrazolium salt, and a divalent metal salt of sufficient type and in sufficient amount to stablize the reagent.

Phenazine derivative/tetrazolium salt reagent systems become unstable at pHs above about 5.5. However, many enzymes, such as glucose dehyrogenase, hexokinase, and glucose-6-phosphate dehyrogenase, optimally function at pHs above 7. Importantly, the inclusion of the divalent metal salt in the reagent stabilizes the phenazine derivative/tetrazolium salt system at pHs from about 5.5 to about 7.8, thereby permitting the optimal functioning of certain enzymes (such as the enzymes mentioned above) in the assay of an analyte. (At pHs above about 7.8, phenazine derivatives begin to decompose.)

The inventive reagent may be provided in solution form or may be incorporated into a film, a fabric mesh, a glass fiber matrix, or a paper matrix. Stabilizing a film with the inventive reag nt is particularly important because ordinary drying of films that include a phenazine

derivative and a tetrazolium salt can cause degradation f the reagent.

The invention also describes assay methods utilizing the inventive reagent.

DETAILED DESCRIPTION OF THE INVENTION

The inventive reagent is useful for the analysis of an analyte (for example, glucose). At a minimum, the reagent includes an enzyme (for example, glucose oxidase), a phenazine derivative (for example, phenazine ethosulfate), a tetrazolium salt (for example, iodonitrotetrazolium chloride (INT) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)), and a divalent metal salt (such as nickel chloride or manganese sulfate). If the reagent is supplied in solution form, then the reagent will also include water. If the reagent is supplied in a film, then a film forming agent (such as hydroxyethylcellulose and polyvinylpropionate, available from BASF and sold under the trademark PROPIOFAN 70D) will be included. (MTT is very sensitive and was not stalilized in a film.) In a film, water is essentially removed from the reagent by drying the film.

In the reagent, the enzyme must be of sufficient type and in sufficient amount to catalyze the reaction of analyte and phenazine derivative. The kind of enzyme will depend upon the analyte being analyzed. For example, if the analyte is glucose, the enzyme may be glucose oxidase. Other exemplary analyte/enzyme combinations are listed below in Table 1.

TABLE 1

Analyte	Enzyme
Glucose	Glucose Dehydrogenase & Diaphorase
Cholesterol	Cholesterol Esterase & Cholesterol Oxidase
HDL Cholesterol	Cholesterol Esterase & Cholesterol Oxidase
Triglycerides	Lipoprotein Lipase, Glycerol Kinase, & Glycerol-3-Phosphate Oxidase
Lactate	Lactate Oxidase
Lactate	Lactate Dehydrogenase & Diaphorase
Lactate Dehydrogenase	Diaphorase
Pyruvate	Pyruvate Oxidase
Alcohol	Alcohol Oxidase
Bilirubin	Bilirubin Oxidase

The amount of enzyme will depend upon the speed desired for the assay (the more enzyme added, the faster the test).

The phenazine derivative employed must be of sufficient type and in sufficient amount to catalyze the reduction of the tetrazolium salt. In the context of this invention, phenazine derivatives are used as electron carriers facilitating the reduction of the tetrazolium salt. Examples of phenazine derivatives that may be used are listed below in Table 2.

TABLE 2

Phenazine Derivative:
Phenazine Ethosulfate
Phenazine Methosulfate
1-Methoxyphenazine Methosulfate

Because the phenazine derivative is a non-depleted catalyst in the assay, less than a molar equivalent of phenazine derivative, relativ to the analyte being measured, is required in the reagent.

The tetrazolium salt employed must be of sufficient type and in sufficient amount to form a colored indicator when reduced. The reduced tetrazolium salt is a formazan, which is a colored indicator. The amount of this colored indicator formed in an assay is correlated to the amount of analyte in the sample being measured. Examples of the tetrazolium salts that may be used in the reagent are listed below in Table 3.

TABLE 3

Tetrazolium Salts:

MTT (if the reagent is in solution form) (Note: nickel chloride is known to chelate MTT (MTT formazan) and cause a wavelength shift in the MTT formazan.)

Iodonitrotetrazolium chloride (INT)

Tetranitro blue tetrazolium chloride (TNBT)

Because there must be at least a molar equivalent of tetrazolium salt with respect to the analyte being measured, a sufficient amount of tetrazolium salt should be included in the reagent to cover the high end of expected concentrations of analyte in the samples being measured.

The focus of this invention is the inclusion of divalent metal salts in the reagent. The divalent metal salts must be of sufficient type and in sufficient amount to stabilize the reagent. Such divalent metal salts include salts with a cation selected from the group consisting of nickel, manganese and magnesium, and an inert anion (an anion that will not chemically react in the assay of the analyte being measured). Specific examples of such divalent metal salts include nickel chloride, manganese sulfate, magnesium sulfate, magnesium chloride, and nickel sulfate. These divalent metal salts may be added individually or in combinations to a reagent to stabilize the reagent. Further, adding a combination of these divalent metal salts to a reagent can sometimes

better stabilize the reagent than addition of a single divalent metal salt.

The divalent metal salt cobalt chloride, which imparts a red color to solutions, can stabilize a liquid reagent that includes TRIS buffer (see Table 4). However, cobalt chloride reacts with HEPES buffer (see Table 4) at pH 7.55. Further, cobalt chloride can not be used in a film (or a fabric mesh, a glass fiber matrix, or a paper matrix) that includes a tetrazolium salt because cobalt chloride forms a blue colored complex with water upon drying, thereby interfering with an assay because formazans are blue colored.

When stabilizing a liquid reagent by the addition of divalent metal salts, the molar ratio of divalent metal salts to phenazine derivative should be at least about 30:1. When this ratio was used in a liquid reagent that included magnesium chloride and phenazine ethosulfate, a liquid reagent that did not include a tetrazolium salt was stable for at least 2 hours under ambient laboratory room lighting and temperature conditions. (When no divalent cation was present, this liquid reagent was stable for less than two hours.) It is believed that stability would be about the same for a liquid reagent that also included a tetrazolium salt because it appears that most of the reagent instability is due to instability of the phenazine derivative. (For example, an aqueous solution that includes a buffer and MTT was stable for at least 3 hours.)

Under the same conditions stated above, when the molar ratios of magnesium chloride to phenazine ethosulfate were 30:1, 60:1, 120:1, and 187:1, the reagents were stable for at least 2 hours, 3 hours, 3 days, and 3 days respectively. (Again, it is believed that reagent stability would be about the same if a tetrazolium salt were included.)

Molar ratios f divalent metal salts to phenazine derivative even higher than 187:1 may be used as long as the divalent metal salts are soluble in the reagent and any necessary pH adjustments to the reagent are made. (For example, molar ratios of nickel chloride to phenazine ethosulfate greater than 30:1 require adjustment of the reagent pH because the addition of nickel chloride lowers reagent pH.) Further, the higher the reagent pH, the greater the molar ratio of divalent metal salt to phenazine derivative needs to be in order to stabilize the reagent. The higher molar ratio of divalent metal salt to phenazine derivative is required at higher pHs because degradation of the phenazine derivative and the tetrazolium salt in the reagent become more pronounced at higher pHs.

When a film, a fabric mesh, a glass fiber matrix, and a paper matrix are being stabilized, the ratio of divalent metal salt to phenazine derivative should be at least about 15:1 when the reagent pH is about 6.8 and at least about 25:1 when the reagent pH is about 7.4. (As with the liquid reagent, the ratio of divalent metal salt to phenazine derivative required to stabilize the film is higher at higher pHs because degradation of the phenazine derivative and the tetrazolium salt in the film, the fabric mesh, the glass fiber matrix, and the paper matrix is more pronounced at higher pHs. Also, the ratios would need to be higher to stabilize a film, a fabric mesh, a glass fiber matrix, and a paper matrix that included MTT. Stabilization of a film, a fabric mesh, a glass fiber matrix, and a paper matrix that included MTT would be easier under humidity conditions of less than about 15%.) Higher ratios of divalent metal salt to phenazine derivative may be used, but no further stabilization is achieved by such higher ratios.

Importantly, the inclusion of the divalent metal salts stabiliz s the ph nazine derivative/tetrazolium

salt reagent at pHs from about 5.5 to about 7.8. (Such films that do not include a divalent metal salt show a formazan blank reaction upon drying the wet film for 15 minutes at about 50°C.) The reagent is stable without the inclusion of a divalent metal salt below pHs of about 5.5. However, above pHs of about 5.5 photochemical degradation of phenazine derivatives and autoreduction of tetrazolium salts become significant. The problem of degradation of phenazine derivatives and tetrazolium salts worsens as the pH increases. It is desirable to conduct assays at pH values above about 5.5 because many enzymes optimally function at pH values above about 5.5. For example, glucose dehydrogenase optimally functions at a pH of about 7.9, and hexokinase and glucose-6-phosphate dehydrogenase optimally function at a pH of about 7.8. Inclusion of the divalent metal salts of the present invention in a reagent make degradation of the phenazine derivative and tetrazolium salt insignificant in the pH range from 5.5 to about 7.8, thereby permitting better performance of assays in that pH range (where certain enzymes function best). Further, it is particularly important to stabilize films that include a phenazine derivative and a tetrazolium salt because degradation of the phenazine derivative and the tetrazolium salt is worsened by the drying process used to form the film.

A buffer is preferably provided in the reagent in order to provide a pH most beneficial to the assay. The buffer should be of sufficient type and in sufficient amount to provide and maintain a pH for the enzyme to function as a catalyst. Table 4 below exemplifies buffers that may be used in the pH range in which they effectively buffer a solution.

TABLE 4

BUFFER	pH RANGE
MES(2-[N-Morpholino]ethanesulfonic acid)	5.5-6.7
BIS-TIS(bis[2-Hydroxyethyl]imino-tris-[hydroxymethyl]-methane)	5.8-7.2
ADA(N-[2-Acetamido]-2-iminodiacetic acid)	6.0-7.2
PIPES(Piperazine-N,N'-bis[2-ethanesulfonic acid])	6.1-7.5
ACES(2-[(2-Amino-2-oxoethyl)-amino]ethanesulfonic acid)	6.1-7.5
BIS-TRIS PROPANE(1,3-bis[tris(Hydroxy-methyl)methylamino]-propane)	6.3-9.5
MOPSO(3-[N-Morpholino]-2-hydroxy-propanesulfonic acid)	6.2-7.6
BES(N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid)	6.4-7.8
MOPS(3-[N-Morpholino]propanesulfonic acid)	6.5-7.9
TES(N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid)	6.8-8.2
HEPES(N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	6.8-8.2
TAPSO(3-[N-tris(Hydroxymethyl) methylamino]-2-hydroxypropane-sulfonic acid)	7.0-8.2
POPSO(Piperazine-N,N'-bis[2-hydroxy-propanesulfonic acid])	7.2-8.5
EPPS(N[2-Hydroxyethyl]-piperazine-N'-3-propanesulfonic acid)	7.3-8.7
TRIS(tris[Hydroxymethyl]amino-methane)	7.0-9.0
TRICINE(N-tris[Hydroxymethyl]-methylglycine)	7.4-8.8
BICINE(N,N-bis[2-Hydroxyethyl] glycine)	7.6-9.0
TAPS(tris[Hydroxymethyl]methylamino-propanesulfonic acid)	7.7-9.1

Examples of a liquid reagent and a film are given below.

Reagent Example 1

A film may be prepared with the following ingredients in the following amounts (total mass of ingredients = 1 kilogram):

Ingredient	Amount in Grams (Unless Otherwise Specified)
0.3 Molar (M) HEPES Buffer (pH = 7.5)	253.3
20% (by weight (wt.)) aqueous composition of various polyoxyethylene ethers and other surface-active compounds, sold under the trademark TRITON X-100, available from Sigma Chemical Company	14.5
manganese sulfate	16.7 (99 milli moles (mmol))
nickel chloride	23.6 (99 mmol)
diatomite, sold under the trademark CELATOM MW25, available from Eagle Picher	157.4
titanium dioxide, available from Kronos as TiO_2 RN 43	148.3
phenazine ethosulfate, available from Research Organics, Inc.	1.3 (3.9 mmol)
3:2:1 (wt.:wt.:wt.) acetone:hexanol: methanol	4.2
50:50 (wt.:wt.) water: polyvinylpropionate (sold under the trademark PROPIOFAN 70D, available from BASF)	104.5
3% (by wt.) methyl cellulose, sold under the trademark TYLOSE MH 2000, available from Hoechst, in 0.3 M HEPES buffer	261.9

Glucose Oxidase (Grade I, available from Biozyme)	1,754.4 kilounits
INT	5.689(11.25 mmol)

The film may be made as follows:

step # 1 - add 20% (by wt.) aqueous TRITON X-100 surfactant to the 0.3 M HEPES buffer (pH = 7.5);
step # 2 - next, add the manganese sulfate and nickel chloride;
step # 3 - next, add titanium dioxide and stir until the resulting mixture is homogeneous;
step # 4 - next, add CELATOM MW25 diatomite;
step # 5 - next, add 3% (by wt.) TYLOSE MH 2000 methyl cellulose in 0.3 M HEPES buffer;
step # 6 - next, add PROPIOFAN 70D;
step # 7 - next, add glucose oxidase;
step # 8 - next, add INT;
step # 9 - next, add 3:2:1 (wt.:wt.:wt.) acetone:hexanol:methanol;
step #10 - centrifuge to remove bubbles;
step #11 - next, stir until contents are homogeneous;
step #12 - next, filter the homogeneous contents through a mesh bag to remove clumps
step #13 - next, stir the filtrate until it is homogeneous;
step #14 - next, coat these contents onto Cronar plastic, providing a coating thickness of 250 micrometers (μm);
step #15 - dry the coating at 45° C for 30 minutes.

Reagent Example 2

An aqueous reagent may be prepared with the following ingredients in the following concentrations:

Ingredient	Concentration (millimolar (mM) unless otherwise specified)
aqueous HEPES BUFFER (pH = 7.3)	100
magnesium chloride	250
phenazine ethosulfate	5
glucose oxidase	1 kilounit/ milliliter (ml) reagent
INT	12

In preparing the above stated aqueous reagent, phenazine ethosulfate and INT should be added last to the reagent.

The assay of an analyte may be conducted by performing the following steps:

a. forming a test sample by combining a fluid sample containing the analyte and the reagent of the present invention;

b. incubating the test sample;

c. spectrophotometrically measuring the incubated test sample; and

d. correlating the spectrophotometric measurement to the concentration of analyte in the fluid sample.

The incubation temperature and time period for incubation will depend upon the assay being conducted. The wavelength of spectrophotometric measurement and the type of spectrophotometric measurement (for example absorbance, transmittance, or reflectance) will depend upon the tetrazolium salt utilized in the reagent and whether spectrophotometric measurements are made of a solution (utilizing a liquid reagent) or a film,

respectively. Examples of specific assay pr cedures utilizing the specifically formulated reagents exemplified above are given below.

ASSAYS FOR GLUCOSE

Assay Example 1

In a cuvet, combine 2.9 milliliters (ml) of the above stated liquid reagent (Reagent Example 2) and 0.1 ml of a liquid unknown sample, containing an unknown amount of from about 0 to about 600 milligrams (mg) glucose per deciliter (dl) of sample, and vortex the resulting contents to mix the liquid unknown sample and liquid reagent. (assay step a. above) Incubate the resulting contents at room temperature for 15 seconds (assay step b. above); then read spectrophotometric absorbance of the incubated, resulting contents at 580 nanometers (nm). (assay step c. above) Compare spectrophotometric absorbance of the incubated, resulting contents with the spectrophotometric absorbance of a standard (that is, the above stated liquid reagent combined with and incubated, as specified above, with a liquid known sample containing a known amount of from about 0 to about 600 mg glucose per dl of known sample), and correlate the spectrophotometric absorbance of the incubated, resulting contents with the concentration of glucose in the liquid unknown sample. (assay step d. above)

Assay Example 2

For glucose analysis performed with the film specified above (Reagent Example 1), add enough liquid unknown sample, containing an unknown amount of from about 0 to about 600 mg glucose per dl of sample, to wet the film. (assay step a. above) Incubate the wetted film for about 30 seconds at room temperature. (assay step b. above) Measure spectrophotometric reflectance of the incubated film at 580 nm. (assay step c. above) Compare measured spectrophotometric reflectance of the

incubated film at 580 nm with the spectrophotometric reflectance of a film treated as above with the excepti n that a liquid known sample (containing a known amount of from about 0 to about 600 mg glucose per dl of sample) was used to wet the film rather than a liquid unknown sample, and correlate the spectrophotometric reflectance of the incubated film to the concentration of glucose in the unknown liquid sample.

The present invention has been disclosed in the above teachings with sufficient clarity and conciseness to enable one skilled in the art to make and use the invention, to know the best mode for carrying out the invention, and to distinguish it from other inventions and from what is old. Many variations and obvious adaptations of the invention will readily come to mind, and these are intended to be contained within the scope of the invention as claimed below.

We Claim:

1. A reagent useful for the analysis of an analyte, comprising:

an enzyme, a phenazine derivative, a tetrazolium salt, a divalent metal salt, and water,

the enzyme being of sufficient type and in sufficient amount to catalyze the reaction of analyte and phenazine derivative,

the phenazine derivative being of sufficient type and in sufficient amount to catalyze the reduction of tetrazolium salt,

the tetrazolium salt being of sufficient type and in sufficient amount to form a colored indicator when reduced, and

the divalent metal salt being of sufficient type and in sufficient amount to stabilize the reagent.

2. The reagent of claim 1, further comprising:

a buffer of sufficient type and in sufficient amount to provide and maintain a pH for the enzyme to function as a catalyst.

3. The reagent of claim 2, wherein the divalent metal salt consists of:

a. a cation selected from the group consisting of nickel, manganese and magnesium; and

b. an inert anion.

4. The reagent of claim 2, wherein the divalent metal salt is selected from the group consisting of nickel chloride, manganese sulfate, magnesium sulfate, magnesium chloride, and nickel sulfate.

5. The reagent of claim 2, wherein the divalent metal salt is cobalt chloride and the buffer is tris(hydroxymethyl)aminomethane.

6. The reagent of claim 2, wherein the divalent metal salt is selected from the group consisting of nickel chloride, manganese sulfate, and nickel sulfate.

7. The reagent of claim 1, wherein the molar ratio of divalent metal salt to phenazine derivative is at least about 30:1.

8. The reagent of claim 3, wherein the molar ratio of divalent metal salt to phenazine derivative is at least about 30:1.

9. The reagent of claim 5, wherein the molar ratio of cobalt chloride to phenazine derivative is at least about 30:1.

10. The reagent of claim 7, wherein the reagent pH is from about 5.5 to about 7.8.

11. The reagent of claim 2, wherein the analyte is glucose, the buffer concentration is about 100 millimolar, the divalent metal salt concentration is about 250 millimolar, the phenazine derivative concentration is about 5 millimolar, the enzyme is glucose oxidase at a concentration of about 1 kilounit per milliliter of reagent, and the tetrazolium salt concentration is about 12 millimolar.

12. A reagent, which is impregnated in a fabric mesh, a glass fiber matrix, or a paper matrix and useful for the analysis of an analyte from a fluid sample, the reagent comprising:

an enzyme, a phenazine derivative, a tetrazolium salt, and a divalent metal salt,

the enzyme being of sufficient type and in sufficient amount to catalyze the reaction of analyte and phenazine derivative,

the phenazine derivative being of sufficient type and in sufficient amount to catalyze the reduction of tetrazolium salt,

the tetrazolium salt being of sufficient type and in sufficient amount to form a colored indicator when reduced, and

the divalent metal salt being of sufficient type and in sufficient amount to stabilize the reagent.

13. The reagent of claim 12, further comprising:

a buffer of sufficient type and in sufficient amount to provide and maintain a pH for the enzyme to function as a catalyst.

14. The reagent of claim 13, wherein the divalent metal salt consists of:

a. a cation selected from the group consisting of nickel, manganese and magnesium; and

b. an inert anion.

15. The reagent of claim 12, wherein the molar ratio of divalent metal salt to phenazine derivative is at least about 15:1.

16. The reagent of claim 12, wherein the molar ratio of divalent metal salt to phenazine derivative is at least about 25:1.

17. The reagent of claim 15, wherein the buffer is of sufficient type and in sufficient amount to provide a pH from about 5.5 to about 7.8 when the fluid sample containing the analyte is added to the reagent impregnated woven fabric mesh, glass fiber matrix, or paper matrix.

18. A film useful for the analysis of an analyte, comprising:

an enzyme, a phenazine derivative, a tetrazolium salt, a divalent metal salt, and a film forming agent,

the enzyme being of sufficient type and in sufficient amount to catalyze the reaction of analyte and phenazine derivative,

the phenazine derivative being of sufficient type and in sufficient amount to catalyze the reduction of tetrazolium salt,

the tetrazolium salt being of sufficient type and in sufficient amount to form a colored indicator when reduced,

the divalent metal salt being of sufficient type and in sufficient amount to stabilize the film, and

the film forming agent being in sufficient amount to form the film.

19. The film of claim 18, further comprising:

a buffer of sufficient type and in sufficient amount to provide and maintain a pH for the enzyme to function as a catalyst when a fluid containing the analyte is added to the film.

20. The film of claim 19, wherein the divalent metal salt consists of

a. a cation selected from the group consisting of nickel, manganese and magnesium; and

b. an inert anion.

21. The film of claim 19, wherein the divalent metal salt is selected from the group consisting of nickel chloride, manganese sulfate, magnesium sulfate, magnesium chloride, and nickel sulfate.

22. The film of claim 19, wherein the divalent metal salt is selected from the group consisting of nickel chlorid , manganese sulfate, and nickel sulfate.

23. The film f claim 18, wherein the molar ratio of divalent metal salt to phenazine derivative is at least about 15:1.

24. The film of claim 18, wherein the molar ratio of divalent metal salt to phenazine derivative is at least about 25:1.

25. The film of claim 20, wherein the molar ratio of divalent metal salt to phenazine derivative is at least about 15:1.

26. The film of claim 20, wherein the molar ratio of divalent metal salt to phenazine derivative is at least about 25:1.

27. The film of claim 23, wherein the buffer is of sufficient type and in sufficient amount to provide a pH from about 5.5 to about 7.8 when a fluid sample containing the analyte is added to the film.

28. A film useful for the analysis of glucose, comprising per kilogram before drying:

a. about 25% (by weight) 0.3 molar N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid buffer;

b. about 1.4% (by weight) of 20% (by weight) aqueous TRITON X-100 surfactant;

c. about 1.7% (by weight) manganese sulfate;

d. about 2.4% (by weight) nickel chloride;

e. about 16% (by weight) CELATOM MW 25 diatomite;

f. about 15% (by weight) titanium dioxide;

g. about 0.1% (by weight) phenazine ethosulfate;

h. about 0.4% (by weight) of 3:2:1 (weight:weight:weight) acetone:hexanol:methanol;

i. about 10% (by weight) of 50:50 (weight:weight) water: PROPIOFAN 70D polyvinylpropionate;

j. about 26% (by weight) of 3% (by weight) TYLOSE MH 2000 methyl cellulose in 0.3 molar N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer;

k. about 1.75×10^6 units glucose oxidase; and

l. about 0.6% (by weight) iodonitrotetrazolium chloride.

29. A method for the assay of an analyte, comprising the steps of:

a. forming a test sample by combining a fluid sample containing the analyte and the reagent of claim 1;

b. incubating the test sample;

c. spectrophotometrically measuring the incubated test sample; and

d. correlating the spectrophotometric measurement to the concentration of analyte in the fluid sample.

30. A method for the assay of an analyte, comprising the steps of:

a. forming a test sample by combining a fluid sample containing the analyte and the reagent of claim 3;

b. incubating the test sample;

c. spectrophotometrically measuring the incubated test sample; and

d. correlating the spectrophotometric measurement to the concentration of analyte in the fluid sample.

31. A method for the assay of an analyte, comprising the steps of:

a. forming a test sample by combining a fluid sample containing the analyte and the reagent of claim 10;

b. incubating the test sample;

c. spectrophotometrically measuring the incubated test sample; and

d. correlating the spectrophotometric measurement to the concentration of analyte in the fluid sample.

32. A method for the assay of an analyte, comprising the steps of:

a. forming a test sample by combining a fluid sample containing the analyte and the film of claim 18;

b. incubating the test sample;

c. spectrophotometrically measuring the incubated test sample; and

d. correlating the spectrophotometric measurement to the concentration of analyte in the fluid sample.

33. A method for the assay of an analyte, comprising the steps of:

a. forming a test sample by combining a fluid sample containing the analyte and the film of claim 20;

b. incubating the test sample;

c. spectrophotometrically measuring the incubated test sample; and

d. correlating the spectrophotometric measurement to the concentration of analyte in the fluid sample.

34. A method for the assay of an analyte, comprising the steps of:

a. forming a test sample by combining a fluid sample containing the analyte and the film of claim 27;

b. incubating the test sample;

c. spectrophotometrically measuring the incubated test sample; and

d. correlating the spectrophotometric measurement to the concentration of analyte in the fluid sample.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06282

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12N 9/96; G01N 33/70
US CL :435/188, 190, 18; 436/73, 164; 422/56
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/188, 190, 18; 436/73, 164; 422/56

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 3,929,580 (Forgione et al.) 30 December 1975, See entire document.	1-34
Y	US, A, 4,427,771 (Misaki et al.) 24 January 1984, See entire document.	1-34
Y	US, A, 4,457,461 (Esders et al.) 15 October 1985, See Col. 10, lines 42-62.	1-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be part of particular relevance
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